Mn\(^{2+}\) and Ca\(^{2+}\) Binding to the Lima Bean Lectins*

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We have purified the two lectins from lima bean (Phaseolus lunatus) and studied the binding of Ca\(^{2+}\) and Mn\(^{2+}\) ions to each. The 128,000-dalton tetrameric lima bean lectin (LBL) and the 240,000-dalton octamer (LBL\(_a\)) were purified by chromatography on Ultrogel and sulfoethyl Sephadex. Using equilibrium dialysis, we have found that demetallized LBL (apo-LBL) will bind either 2 Mn\(^{2+}\) or 4 Ca\(^{2+}\) ions. The Ca\(^{2+}\) binding is strongly cooperative while the Mn\(^{2+}\) binding is not. If apo-LBL is presaturated with Mn\(^{2+}\) (Mn\(^{2+}\)-LBL), it will bind only 2 Ca\(^{2+}\) ions in a noncooperative manner. Apo-LBL presaturated with Ca\(^{2+}\) (Ca\(^{2+}\)-LBL\(_a\)) does not bind Mn\(^{2+}\). The metal stoichiometry for LBL\(_a\) is double that for LBL, under all of the above conditions.

Lectins have received considerable attention in recent years, mainly because of their ability to bind to cell surfaces. This binding is of interest because it can yield information about the composition of the cell surface and because it can sometimes lead to an alteration of cellular activity, often inducing mitogenesis. This mitogenic property of some lectins has been shown to require divalent metal ions for full activity (Galbraith and Goldstein, 1970, 1972b). The lectins contain significant amounts of Mn\(^{2+}\) and Ca\(^{2+}\) when isolated and after purification (Galbraith and Scheinberg, 1970, 1972a) have reported stoichiometries of 0.7 to 1.1 mol of Mn\(^{2+}\) and 4 to 7 mol of Ca\(^{2+}\)/mol of LBL. These data, in conjunction with the sugar stoichiometry, raise some interesting questions with respect to the functional structure of the lima bean lectins. Apparently "half-of-the-sites" sugar binding has been observed in several lectins (Goldstein and Hayes, 1978); however, in each case, subunit heterogeneity has been subsequently demonstrated.

We have developed a new purification procedure using salt fractionation, gel chromatography on Ultrogel, and ion exchange chromatography. This method is relatively simple and inexpensive and yields LBL and LBL\(_a\) of high purity and in sufficient quantity to permit physical studies. Using protein purified in this manner we have obtained antisera against both LBL\(_a\) and LBL, and detected complete cross-reactivity and lines of identity on double immunodiffusion plates with both lectins. We have compared the circular dichroism spectra of LBL\(_a\) and LBL, and we have conducted extensive metal binding studies with Ca\(^{2+}\) and Mn\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Materials—**\(^{44}\)Ca was purchased from New England Nuclear and \(^{55}\)Mn from ICN Pharmaceuticals. Other divalent metal ions were Spectro grade from Johnson Matthey Chemicals, Ltd. Mops and sulfoethyl (SP)-Sephadex were from Sigma and Ultrogel AcA 34 was obtained from LKB. Lima beans were Carolina or Sieva type purchased from Burpee Seed Co.

**Lima Bean Lectin Purification—**All solutions contain 0.03% NaN\(_3\), and 10 \(\mu\)M dithiothreitol and are at 5°C unless otherwise indicated. Lima beans (250 g) were ground into a fine meal in a Waring Blendor. The meal was suspended in 2 liters of 0.1 M phosphate, pH 6.8, and stirred for 1 h at room temperature. The suspension was filtered through cheesecloth and the filtrate was centrifuged at 30,000 \(\times\) g, 5°C, for 30 min. The supernatant was retained and 280 g/liter (NH\(_4\))\(_2\)SO\(_4\) were added (45% saturation). After stirring for 2 h, the pH was adjusted to 5.0 and the solution was centrifuged at 30,000 \(\times\) g for 40 min. The supernatant was retained and 32 g/liter (NH\(_4\))\(_2\)SO\(_4\) was added (50% saturation). This solution was stirred overnight and
centrifuged at 45,000 × g for 40 min. The pellet was dissolved in 0.5 M NaCl, 0.01 M Mops, pH 7.0, and dialyzed against 4 liters of this same buffer overnight. This 45 to 50% salt fraction was filtered using a type HA 45-µm Millipore filter and applied to an Ultrogel AcA 34 column (5 × 174 cm) flowing at 30 ml/h. Elution was carried out with 0.5 M NaCl, 0.01 M Mops, pH 7.0. Fractions were collected and absorbance at 280 nm was monitored. Fractions from the major peaks were tested for specific agglutination of human type A red blood cells. Agglutination assays were conducted in microtiter plates using a 3% suspension of freshly washed red cells. Fractions from the active peaks were pooled, concentrated using an Amicon Diaflo apparatus (with a UM-10 membrane, and analyzed by gel electrophoresis at pH 4.3 (Heisfeld et al., 1962). Occasionally, the LBL* peak contained minor contamination, in which case it was dialedyzed into 0.1 M NaCl, 0.01 M acetate, pH 5.0, and applied to a column of SP-Sephadex (2.5 × 50 cm), and eluted with a linear gradient of NaCl. The pure, active fractions were pooled, concentrated, and dialedyzed into 0.5 M NaCl, 0.01 M Mops, pH 7.0.

The purity of the lima bean lectins has been verified by gel electrophoresis at pH 4.3 and 8.3 (Brewer and Ashworth, 1969), SDS gel electrophoresis (Laemmli, 1970), sedimentation equilibrium, and antibody precipitation in agarose gels. The protein concentration was determined by absorbance at 280 nm using ε280, = 12.3 (Gould and Scheinberg, 1970).

Circular Dichroism and Sedimentation Equilibrium—Circular dichroism was performed using a JASCO J-40A spectrometer. A Beckman Spinco model E was used to obtain sedimentation equilibrium data. The lima bean lectins were demetallized by dialysis against 0.1 M EDTA, 0.5 M NaCl, pH 7.0, over a 7-day period. The EDTA was removed by dialysis (8 × 250 ml over 6 days) against 0.5 M NaCl, 0.01 M Mops, pH 6.7, which was prepared from 1 ml NaCl demetallized by two passages over a Chelex 100 column. The demetallized lectins were tested for divalent metal contamination before and after experiments. Atomic absorption spectroscopic determinations (Perkin-Elmer model 303) of Mn++, Ni++, Co++, and Zn++ showed that total contamination from these metals did not exceed 1% (with respect to 30,000dalton subunits) and Ca++ contamination never exceeded 4%. This demetallization procedure yields lectin which can be fully reactivated as determined by agglutination assays.

Metal Binding Studies—All metal binding data were obtained by equilibrium dialysis using 45Ca and 54Mn. Experiments were performed by placing 1 ml of demetallized lima bean lectin in a dialysis bag and immersing in 4 ml of buffer containing radioactive metal in a polypropylene culture tube. The samples were incubated at 5°C for 3 to 5 days with gentle agitation using a rotary shaker. For each experiment, control samples without radioactive metal were prepared in order to determine the final protein concentration, metal contamination, and activity. 45Ca was counted in Bray’s (Bray, 1960) solution. 54Mn was counted using a Beckman Gamma 4000.

RESULTS

Purification of the Lima Bean Lectins—The results of our purification technique are summarized in Table I and Fig. 1. Ultrogel AcA 34 chromatography consistently yielded LBL4 without detectable contamination. Fractions pooled from the

Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Titer</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µl</td>
<td>mg/mg</td>
<td></td>
<td>units/ml</td>
</tr>
<tr>
<td>Crude extract</td>
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<td>39.8</td>
<td>32</td>
<td>1.3</td>
</tr>
<tr>
<td>pH 5 supernate</td>
<td>1.6</td>
<td>30.4</td>
<td>32</td>
<td>1.7</td>
</tr>
<tr>
<td>45-50 salt cut</td>
<td>0.07</td>
<td>0.32</td>
<td>4096</td>
<td>550</td>
</tr>
<tr>
<td>Ultrogel LBL4 peak</td>
<td>0.09</td>
<td>0.12</td>
<td>512</td>
<td>380</td>
</tr>
<tr>
<td>Ultrogel LBL4 peak</td>
<td>0.04</td>
<td>0.025</td>
<td>1024</td>
<td>1600</td>
</tr>
<tr>
<td>SP-Sephadex LBL4 peak</td>
<td>0.025</td>
<td>0.015</td>
<td>1024</td>
<td>1700</td>
</tr>
</tbody>
</table>

* Titer is expressed as the inverse of the maximum dilution giving agglutination of human type A red blood cells.

FIG. 1. Elution profiles of the lima bean lectins. A, elution profile from Ultrogel AcA 34. Thirty-five milliliters of the 45 to 50% salt fraction was applied to a column (5 × 174 cm) and eluted at 30 ml/h at 5°C with 0.5 M NaCl, 0.01 M Mops, pH 7.0 (6 ml/fraction). B, elution profile from SP-Sephadex. Forty milliliters of the Ultrogel LBL4 peak was concentrated to 10 ml and dialyzed into 0.1 M NaCl, 0.01 M acetate, pH 5.0. This was applied to an SP-Sephadex (2.5 × 50 cm) column and eluted at 20 ml/h with a linear gradient of NaCl increasing to 0.6 M NaCl (8.5 ml/fraction).

FIG. 2. Gel electrophoresis of the lima bean lectins. Rows A to C are pH 4.3 gels with 7.5% acrylamide run at 6 mA/tube for 3 h. D is an SDS-gel (12.5% acrylamide) with β-mercaptoethanol. A, 40 µg of LBL4 from Ultrogel peak. B, 40 µg of protein from Ultrogel LBL4 peak. C, 40 µg of LBL4 from SP-Sephadex. D, 15 µg of purified LBL4 plus 15 µg of purified LBL4.

Ultrogel-LBL4 peak sometimes showed minor contaminants on pH 4.3 gels (Fig. 2). Chromatography of concentrated LBL4 fractions on SP-Sephadex yielded pure LBL4. No contamination of the purified lima bean lectins was detected by gel electrophoresis at pH 4.3 (5 and 7.5% acrylamide) and pH 8.3, SDS-gel electrophoresis, sedimentation velocity and equilibrium, and immunodiffusion against antisera in agarose gels.

Gould and Scheinberg (1970) determined a lima bean lectin subunit molecular weight of 31,000 based on SDS-gel electrophoresis. They also estimated the molecular weights of LBL4 and LBL5 at 138,100 and 269,000, respectively, using sedimentation velocity. Galbraith and Goldstein (1972a) also used sedimentation velocity to estimate the tetramer molecular weight at 124,400 and the octamer molecular weight at 247,100. Using sedimentation equilibrium, we have determined the LBL4 molecular weight to be 120,800 ± 1900. Using SDS-gel electrophoresis (Laemmli, 1970) with lysozyme (14,400), concanavalin A (25,500, 13,000, 12,000), α-amylase (48,500), creatine kinase (40,500), and pyruvate kinase (57,000)
Metal Binding to Lima Bean Lectin

**Fig. 3 (left).** Scatchard plot of Mn²⁺ binding to apo-LBL₄. The ratio of bound Mn²⁺ ions to total LBL₄ molecules is represented by \( v \) and Mn²⁺ is the free Mn²⁺ concentration. LBL₄ concentration was 1.8 mg/ml.

**Fig. 4 (center).** Scatchard plot of Ca²⁺ binding to apo-LBL₄. The ratio of bound Ca²⁺ ions to total LBL₄ molecules is represented by \( v \) and Ca²⁺ is the free Ca²⁺ concentration. LBL₄ concentration was 3.4 mg/ml. The curve is hand-drawn to best fit the data from this and other similar experiments.

**Fig. 5 (right).** Scatchard plot of Ca²⁺ binding to Mn²⁺-LBL₄. Ca²⁺ and \( v \) are as previously defined. LBL₄ concentration was 3.0 mg/ml. Mn²⁺-LBL₄ was prepared by preincubating apo-LBL₄ in 3 × 10⁻⁴ M Mn²⁺ for 2 h. The experiment was conducted in 3 × 10⁻⁴ M Mn²⁺.

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**TABLE II**

*Metal binding to the lima bean lectin*

All experiments were conducted at pH 6.7 and 5°C.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Conditions</th>
<th>Stoichiometry</th>
<th>Association constant × 10⁻⁶ M⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn⁺⁺</td>
<td>Apo-LBL₄</td>
<td>2.0 ± 0.1</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>Apo-LBL₄</td>
<td>3.9 ± 0.2</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>Mn⁺⁺-LBL₄</td>
<td>2.0 ± 0.1</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>Ca⁺⁺-LBL₄</td>
<td>No binding detectable</td>
<td></td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>Apo-LBL₄</td>
<td>3.7 ± 0.2</td>
<td>N.D.²</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>Apo-LBL₄</td>
<td>8.0 ± 0.4</td>
<td>N.D.²</td>
</tr>
<tr>
<td>Ca⁺⁺</td>
<td>Mn⁺⁺-LBL₄</td>
<td>4.1 ± 0.7</td>
<td>N.D.²</td>
</tr>
<tr>
<td>Mn⁺⁺</td>
<td>Ca⁺⁺-LBL₄</td>
<td>No binding detectable</td>
<td></td>
</tr>
</tbody>
</table>

² Unless otherwise indicated, the stoichiometry and association constants were determined from the intercepts and slopes of Scatchard plots using LBL₄, molecular weight = 120,800, LBL₄ molecular weight = 241,600.

₆ Apo-LBL was prepared as described in the text.

 Errors shown are standard deviations calculated from at least three separate experiments.

 Stoichiometry was determined by equilibrium dialysis using saturating amounts of metal.

 * This binding constant was estimated at half-saturation.

 † Mn⁺⁺- or Ca⁺⁺-LBL₄ was prepared by incubation of apo-LBL₄ in 3 × 10⁻⁷ M Mn⁺⁺ or Ca⁺⁺ before and during the experiment.

 N.D.², N.D., not determined.

as standards, we have estimated the lima bean lectin subunit molecular weight at 29,000.

**Comparison of LBL₄ and LBL₈**—We have obtained antisera to both LBL₄ and LBL₈ and used them to compare the two agglutinins. Immunodiffusion experiments showed that antisera to either LBL₄ or LBL₈ reacted equally well with either lectin and lines of identity were observed in all cases.

We have also examined the far UV circular dichroism spectra of LBL₄ and LBL₈ and found them essentially identical.

**Metal Binding to the Lima Bean Lectins**—Scatchard analysis (Scatchard, 1949) of Mn⁺⁺ binding to apo-LBL₄ showed that it bound 2 Mn⁺⁺ ions with an affinity of approximately 1.8 × 10⁵ M⁻¹ (Fig. 3). Apo-LBL₄ bound 4 Ca⁺⁺ ions and the binding was strongly cooperative (Fig. 4). A binding constant of 4 × 10⁶ M⁻¹ was estimated at half-saturation and Hill plots of the data gave slopes consistently greater than 2. If apo-LBL₄ was presaturated with Mn⁺⁺ (Mn⁺⁺-LBL₄), it bound only 2 Ca⁺⁺ ions and the binding was noncooperative with an association constant of 1.2 × 10⁶ M⁻¹ (Fig. 5). Apo-LBL₄ presaturated with Ca⁺⁺ (Ca⁺⁺-LBL₄) showed no detectable Mn⁺⁺ binding.

Insufficient quantities of protein precluded detailed Scatchard analysis of metal binding to LBL₈. We have determined Mn⁺⁺ and Ca⁺⁺ stoichiometry for the octameric lima bean lectin by equilibrium dialysis using saturating amounts of metal. The results are summarized in Table II. The LBL₈ metal stoichiometry was double that for LBL₄. Either 4 Mn⁺⁺ or 8 Ca⁺⁺ ions bound to apo-LBL₈, 4 Ca⁺⁺ ions bound to Mn⁺⁺-LBL₈, and no Mn⁺⁺ bound to Ca⁺⁺-LBL₈.

**DISCUSSION**

In agreement with Galbraith and Goldstein (1972b), we have found the Gould and Scheinberg (1970) technique unsatisfactory in our hands. The alternate method employed by Galbraith and Goldstein (1970), while yielding ample quantities of pure lectin, involves both affinity and recycling gel chromatography. The affinity chromatography step requires large amounts of the expensive sugar, N-acetyl-D-galactosamine. Our technique yields pure LBL₄ in one chromatographic step, and with some refinement we feel that pure LBL₄ will also be consistently obtainable without the additional ion exchange chromatography step.

Our comparisons of LBL₄ and LBL₈ support the proposal that they are simply different aggregation states of the same types of subunits. Galbraith and Goldstein (1972a) had previously shown that antisera to LBL₄, cross-reacted with LBL₈. Having antibody to only the tetramer, they could not rule out the possibility that LBL₈ contains determinants not found on LBL₄. Having obtained antisera to both lectins and our results support the antigenic identity of the two lectins. Additionally, our preliminary circular dichroism observations indicate that LBL₄ and LBL₈ are conformationally very similar.

One interesting and surprising property of the lima bean
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Lectins is their apparent "half-of-the-sites" sugar binding. LBL4 is thought to be composed of four identical subunits but binds only 2 N-acetyl-D-galactosamine molecules (Bessler and Goldstein, 1974). LBL4 has eight subunits and binds four sugars. Our metal binding results are consistent with the saccharide stoichiometry. LBL4 binds 2 Mn" ions and LBLX binds 4. Although the total Ca'+ stoichiometry for each is equal to the number of subunits, the cooperativity and the observation that half of the Ca"+ sites can be blocked by Mn' suggests that there may be two sets of nonidentical, interacting Ca' sites. Thus, with respect to both sugar and metal binding, LBL4 behaves as a dimer and LBLX as a tetramer. This behavior may be related to the fact that the 30,000-dalton subunits are actually linked together by disulfide bridges to form 60,000-dalton subunits (Gould and Scheinberg, 1970).

The simplest model to fit the metal binding data is the following. Each 60,000-dalton subunit may contain two different metal sites, one able to bind either Ca'+ or Mn" and the other able to bind only Ca'+. When the first site is filled with either metal, the second site now has a greater affinity for Ca'+. This is supported by the observed cooperative binding of Ca' to apo-LBL4 and the noncooperative but greatly enhanced binding of Ca' to Mn"-LBL4. The association constant for Ca' binding to apo-LBL4 is approximately 4 x 10^10 M^-1 at half-saturation. The Ca' binding constant for Mn"-LBL4 is 30 times as great. Indeed, near saturation the Ca' affinity of apo-LBL4 approaches that of Mn"-LBL4.

The "half-of-the-sites" behavior of the lima bean lectins can most reasonably be explained by one of the following three possibilities. Either there is true negative cooperativity between the 30,000-dalton subunits; two or more subunits form a binding site; or the subunits are not actually identical. Future work will be aimed at distinguishing between these possibilities. A more complete understanding of the metal and sugar binding behavior of the lima bean lectins will require better characterization of these proteins. The answers may help to explain the different biological properties of these two lectins.

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